Exact Mass Measurements Using a 7 Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer in a Good Laboratory Practices-Regulated Environment

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Fourier transform ion cyclotron resonance mass spectrometry has been found to produce reliable exact mass measurements using two different internal calibration methods. For these measurements, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) were utilized both individually and in tandem. For internal calibration with a co-dissolved polyethylene glycol standard, measurements of 41 compounds resulted in an average absolute mass determination error of 0.7 ppm, with a standard deviation of 0.9 ppm. For comparison, internal calibration was effected through the simultaneous use of ESI and MALDI, with the former being used for the introduction of analyte ions and the latter for formation of polymethylmethacrylate calibrant ions. This technique led to mass measurements with an average absolute error of 0.8 ppm and a standard deviation of 1.0 ppm. In addition, exact mass measurements of tandem mass spectrometry fragment ions were made for 35 compounds using external calibration with a single internal mass standard. The observed average absolute error was 0.7 ppm with a standard deviation of 1.0 ppm. (J Am Soc Mass Spectrom 1999, 10, 1291–1297) © 1999 American Society for Mass Spectrometry

The pharmaceutical industry is regulated by the Food and Drug Administration and operates under standard Good Laboratory Practices (GLP's) [1]. Under the GLP's, no mass spectrometric data obtained may be discarded. Furthermore, no data may even be disregarded unless they can be defined as outliers for an instrument which has been well characterized. Thus, a mass spectrometric technique must exhibit not only high performance, but also good reliability. This is definitely the case when using mass spectrometry (MS) to make exact mass measurements. Fourier transform ion cyclotron resonance (FTICR) [2, 3] and magnetic sector instruments [4, 5] have been utilized in the past for obtaining exact mass measurements with very low reported error(s). One of the most complete statistical analyses was conducted by Sack et al. [6] in developing a methodology for obtaining exact mass measurements on a sector instrument. Furthermore, the applicability of FTICR for these measurements in the pharmaceutical industry has been enhanced through the recent incorporation of MALDI [7–10] (matrix-assisted laser desorption/ionization) and ESI [11–13] (electrospray ionization), which facilitate the softer ionization (lower fragmentation) of larger organic and biological molecules.

In obtaining reliable exact mass measurements via FTICR MS, many factors are important. These factors may be divided into two general categories: (1) experimental conditions and (2) data processing. In FTICR, ions are introduced into an analyzer cell which is positioned in the central, homogeneous region of a high magnetic field. In this region, the ions undergo cyclotron (circular) motion about the magnetic field axis. The angular frequency of this motion (ω) is observed to be directly proportional to the magnetic field ($\mathbf{B}$) strength and inversely proportional to the mass/charge (m/z) ratio of the ions, as shown in eq 1.

$$\omega = z\mathbf{B}/m \quad (1)$$

In addition, the ions must be contained along the magnetic field axis in order to trap them in the ICR analyzer cell; therefore, two trapping plates, perpendicular to the z axis, are used for this purpose. However, eq 1 fails to account for the presence of the electric field produced by these plates. Thus, a number of equations have been advanced to correct eq 1 for the presence of

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the "trapping" field and the effect of space charge on ion motion.

Processing of FTICR/MS data requires calibration, and thus much work has been done with regard to the development of calibration equations for use with FTICR [2, 14]. The most commonly employed equation has two terms (eq 2) and was introduced by Ledford et al. [15]. The aforementioned space-charge effects can arise from various sources [15–19]. The nature of these effects varies for individual ion packets of different m/z; however, their magnitude can be approximated by considering the relative ion intensities. This approach was utilized by Brown and Smith [20], who have derived an equation (eq 3) which contains an ion intensity term. For these two equations, m/z represents the mass to charge ratio for the ion, V_T is the analyzer cell trapping voltage, ν is the ion cyclotron frequency, A is the magnetic field term coefficient, B is the electric field term coefficient, C is a coefficient in the intensity term, and I is the intensity (peak height) of each individual point in the calibration:

$$m/z = A/\nu + |V_T|B/\nu^2 \quad (2)$$

$$m/z = A/\nu + |V_T|B/\nu^2 + I|V_T|C/\nu^2 \quad (3)$$

In the absence of calibrant ions (for internal calibration), external calibration from another data file may be used for exact mass measurements. Furthermore, a good (stable) superconducting magnet may be expected to have very little frequency drift over several days (or even weeks). Thus the magnetic field term A may remain relatively constant (within 1–2 ppm) during that period of time. However, this is not necessarily the case with the electric field term, B, which can vary for each experiment (even using a constant trapping potential) due to fluctuations in the ion population of the cell, energy of the ions, and the necessity of variable excitation conditions for optimal ion detection. Furthermore, the cleanliness of the cell over a period of time can affect the electric field as well. Therefore, the use of eqs 2 and 3 for external calibration may result in some nonnegligible mass error (>2 ppm). In order to improve upon this external calibration, an adjustment of the electric field term may be required. For this adjustment, a single-point internal lock mass may be used. This type of approach can be used when broadband calibration is required but no multipoint calibrant is present. For instance, this method can be used for exact mass measurements of fragment (successor) ions by using any remaining parent (precursor) ion as an internal lock mass.

Both external and internal calibration have been utilized with FTICR. Furthermore, although internal calibration has typically produced greater accuracy, external methods usually allow for easier sample preparation and thus, higher throughput. The recent development of "multiple ionization techniques simultaneously (MITS) [21]" by Finnigan FTMS has provided a possible means for producing exact mass measurements with both high mass accuracy as well as high throughput. In this procedure, the calibrant and analyte ions are introduced into the cell separately by ESI and MALDI; therefore, it is possible that the ion-cloud distributions and energies for these ions could be different to the extent that it could affect the calibration. Finally, before FTICR can be used (with any of the aforementioned calibration methods) in a GLP-regulated environment, it has to be fully characterized in order to define the normal statistical deviation in error for exact mass measurements via a standardized protocol.

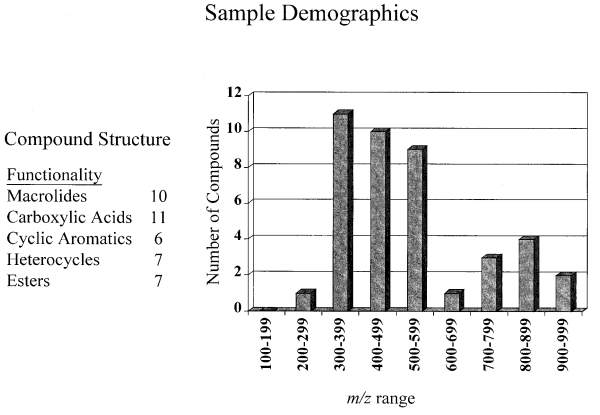

Figure 1. Distribution of samples examined in the study categorized according to m/z and functional group.

In this work, multiple exact mass measurements were conducted for over 40 compounds (Figure 1) in a systematic study designed to fully characterize a 7 tesla FTICR mass spectrometer for use in a GLP-regulated pharmaceutical environment. Furthermore, both MITS and standard internal calibration (internally dissolved standard) were utilized to obtain these measurements in a comparative study. Finally, exact mass measurements of fragment ions were obtained for 35 compounds using both external calibration and external calibration with an internal lock mass.

Experimental

In all of these experiments, a Finnigan FTMS (Madison, WI) Newstar T-70 Fourier transform ion cyclotron resonance mass spectrometer was used in conjunction with an Oxford 7 tesla superconducting magnet and a Finnigan dual cell trap. This system was also equipped with an Analytica of Branford (Branford, MA) external electrospray ionization source. All samples examined in this study had been submitted for routine exact mass analysis. They were introduced via ESI at a flow rate of 3 μL/min from a solution of 1:1 methanol/H_2O with 0.1% acetic acid (AcOH) or 1:1 acetonitrile/H_2O with 0.1% AcOH. For the conventional internal calibration experiments, polyethylene glycol (PEG) was added to

Table 1. Mass spectrometry exact mass measurements as a function of internal calibration—observed errors[a]

Calibration method	Absolute mean error	Standard deviation	rms error
Internal calibration	0.4 mmu (0.7 ppm)	0.5 mmu (0.9 ppm)	0.5 mmu
External calibration	3.1 mmu (5.2 ppm)	5.3 mmu (7.4 ppm)	5.5 mmu
External calibration with a single-point internal lock mass	0.4 mmu (0.8 ppm)	0.6 mmu (1.0 ppm)	0.6 mmu

[a]123 measurements were made using 41 compounds (three measurements/compound).

the solution (as the internal standard) along with the analyte in an approximate ratio of 2:1. In most cases, this had to be estimated due to the extremely small quantities of sample submitted by the customer. Either PEG 300, 400, 500, 600, or 900 was used for each of these experiments depending on the mass of the analyte [the appropriate mass of calibrant was chosen so that at least one internal standard point was present on the higher mass (lower frequency) side of the analyte peak].

Desolvation of the spray was effected through the use of a countercurrent drying gas, N_2, which was heated to 325 °C. The ions were passed through a quartz capillary with a front side voltage of −5000 V and an exit voltage of approximately 100 V. The ions were then directed through a skimmer at 65 V and a hexapole ion guide. Next, after leaving the ESI source, the ions were passed through an ion transfer region (Ultrasource) via a series of acceleration and deceleration ion optics to the low pressure region of the instrument containing the dual cell. The ions were finally trapped in the analyzer cell using a 6.6 V potential (applied to both trapping plates) following an argon pulse to prevent z axis loss of the ions. The trapping potential was then reduced to 0.5 V, and the ions were detected using broadband rf chirp excitation. Data sets containing 512K points were acquired, and coaddition of multiple transients was used only when necessary to provide adequate signal/noise. The data were apodized using a Hamming function and were zerofilled once prior to Fourier transformation. The resolution of both the calibrant and analyte peaks in all of the experiments ranged from 25,000 to 60,000 (FWHH). For all of the data processing discussed in this manuscript, Finnigan FTMS's Odyssey software was utilized.

For the internal calibration experiments via simultaneous ESI and MALDI (MITS), the calibrant ions were introduced by MALDI, immediately followed by introduction of analyte ions via external ESI as discussed previously. For these experiments, polymethylmethacrylate (PMMA) was used as the calibrant. The PMMA was mixed with 2,5-dihydroxybenzoic acid (DHB) in a ratio of 1:5000 PMMA/DHB. The laser irradiation was conducted with the sample probe tip positioned adjacent to the center of the source trap plate. Following the N_2 laser pulse, a 110 μs ion drift time was allowed for transmission of the PMMA ions through the source cell, the conductance limit (separating the source and analyzer cells), and into the analyzer cell. Subsequently, the ions were trapped in the cell prior to introduction of the analyte ions via ESI. Detection conditions were as discussed above for the conventional internal calibration.

Collisionally induced dissociation (CID) studies were conducted for 35 of the 41 compounds discussed above with exact mass measurements produced for the observed fragment ions. For these studies, either on-resonance rf excitation or SORI [22] (sustained off-resonance irradiation) was utilized. The structural characteristics of each individual compound determined which technique was used. In general, on-resonance excitation was used more often with species of lower m/z (<600) while SORI was used primarily with compounds of higher m/z (>600). With on-resonance excitation, a 100 μs excitation pulse was used while a 300 ms excitation pulse was used in SORI experiments. Prior to CID, the precursor ions were isolated via SWIFT [23] (stored waveform inverse Fourier transform) ejection. Subsequently, a 25 ms pulse of argon was introduced into the analyzer cell resulting in a maximum pressure of 1×10^{-7} torr during excitation and a 3 s reaction period was used following excitation. Again, 512K data sets were acquired with co-addition of transients when necessary to improve signal to noise.

External calibration of the data was performed using PMMA (as the standard) in order to obtain the exact mass measurement. Although the magnet used in these studies was found to be very stable (only 1–2 ppm drift over 2 weeks), the calibration files were generated on the same day as the analyte data to minimize any error resulting from magnetic field drift. In addition, a second set of measurements was acquired by using a single-point internal lock mass in order to better optimize the electric field term (B) in eq 2 (for the experimental conditions present in the cell during CID). In this case, the remaining parent ion signal was utilized as the internal lock mass, as its mass had been confirmed previously by the exact mass measurement discussed above. Finally, it is important to note that the same trapping voltage and excite (for detection) conditions were used for the CID experiment and to acquire the external calibration data.

Results and Discussion

Study of Calibration Methods

The primary focus of this project was to develop a reliable methodology for obtaining exact mass measure-

Table 2. Mass spectrometry exact mass measurements as a function of internal calibration—observed errors

Calibration method	Absolute mean error	Standard deviation	rms error
Conventional internal calibration[a]	0.3 mmu (0.7 ppm)	0.4 mmu (0.9 ppm)	0.4 mmu
Internal calibration via dual ionization methods[b]	0.4 mmu (0.8 ppm)	0.6 mmu (1.0 ppm)	0.6 mmu

[a]Conventional method: 69 measurements were made using 23 compounds (three measurements/compound).
[b]Dual ionization method: 54 measurements were made using 18 compounds (three measurements/compound).

ments of both precursor (MS) and product (MS/MS) ions with high accuracy and precision using FTICR MS in a GLP-regulated environment. In this study, three exact mass measurements were acquired for 41 compounds using three different approaches. First, internal calibration was used following two different procedures: the first involved a more conventional approach which utilized ESI to introduce the analyte along with a co-dissolved internal standard, PEG, and the second utilized dual ionization methods simultaneously to introduce both the calibrant (PMMA) and sample ions. For both of these approaches, eq 3 was utilized for calibration of the data. The resulting exact mass measurements from these combined approaches exhibited errors which had a mean absolute error of 0.7 ppm with a standard deviation of 0.9 ppm (Table 1). The minimum error observed for the 123 total measurements was 0.0 ppm while the maximum error was found to be 2.6 ppm. Finally, of the 123 measurements, only five mass measurements had errors >2 ppm.

External calibration was also used to determine the exact mass of these 41 compounds (internal calibrant ion signal present was not used) using eq 3. Again, three measurements were made for each compound using the same data discussed above where conventional internal calibration was used. On a daily basis, prior to sample analysis, an external calibration file was generated using PEG as the calibrant. The resulting exact mass measurements were found to have an absolute mean error of 5.2 ppm with a standard deviation of 7.4 ppm.

Finally, a third set of exact mass measurements were made from this set of spectrometric data; however, in this case, a single-point internal lock mass was used (with eq 2) in order to better optimize the electric field term. The magnetic field term (A) was obtained from the external calibration and kept constant. One of the internal calibrant peaks adjacent (within 100 kHz) to the analyte peak was utilized as the lock mass. The proximity of the frequency of the calibrant peak (to the analyte peak) seemed to be a factor. Furthermore, it was also observed that better results were usually obtained (lower errors in mass measurement) when the selected point was higher in mass (lower in frequency) than the analyte. Although this unexplained effect was found to be negligible with frequency differences less than 100 kHz, it appeared to be much more pronounced with larger frequency differences. This internal adjustment resulted in a substantial improvement in the mass measurements. The absolute mean mass error was reduced to 0.8 ppm while the standard deviation fell to 1.0 ppm. As will be shown below, this type of approach was also useful for obtaining exact mass measurements of fragment ions following MS/MS.

As mentioned above, exact mass measurements via internal calibration were obtained using two different techniques. The first utilized a co-dissolved internal standard while the second made sequential use of MALDI and ESI (MITS) to introduce the calibrant, PMMA, and the analyte respectively for subsequent simultaneous detection of both sets of ions. Of the aforementioned 41 compounds examined via internal calibration, 23 (69 measurements) were randomly selected for mass determination using the conventional approach while the remaining 18 (54 measurements) were obtained using the dual ionization method (Figure

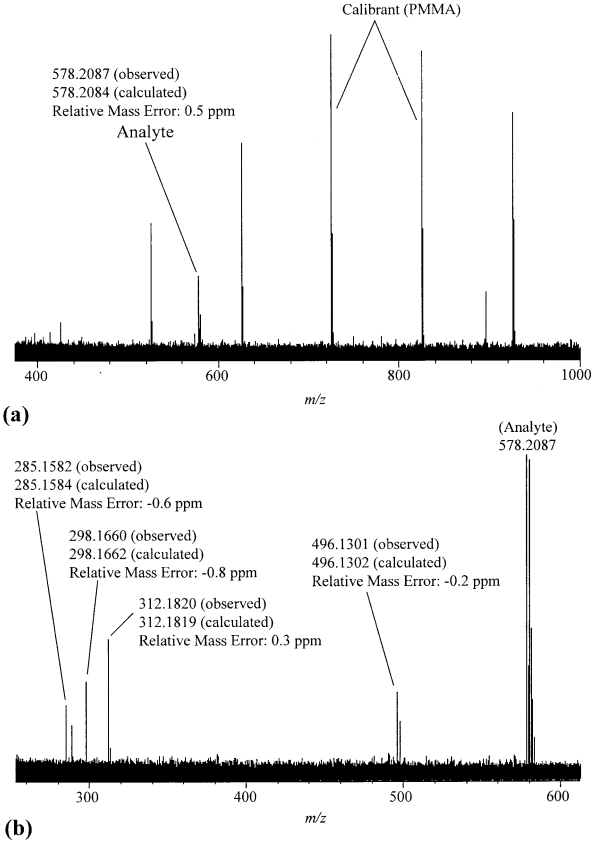

Figure 2. (**a**) Exact mass measurement of an analyte ion observed at m/z 578 using MITS for internal calibration with PMMA as calibrant. (**b**) Exact mass measurements of the product ions produced from the fragmentation of the analyte ion using CID.

Table 3. MS/MS exact mass measurements—observed errors[a]

Calibration Method	Absolute mean error	Standard deviation	rms error
External calibration	1.7 mmu (4.0 ppm)	2.4 mmu (4.8 ppm)	2.8 mmu
External calibration with a single-point internal lock mass	0.3 mmu (0.7 ppm)	0.5 mmu (1.0 ppm)	0.5 mmu

[a]105 measurements were made using 35 compounds (three measurements/compound).

2a). The two methods produced results which were comparable. The standard approach produced an absolute mean error of 0.7 ppm with a corresponding standard deviation of 0.9 ppm while use of the dual ionization procedure resulted in an absolute mean error of 0.8 ppm with a standard deviation of 1.0 ppm.

Most of the compounds analyzed in the previously mentioned mass spectrometry exact mass experiments produced fragment ions via MS/MS (35 of the 41) and exact mass measurements of these ions were obtained. For these exact mass measurements, external calibration was used in conjunction with calibration eq 3. The resulting mass measurements exhibited an absolute mean error of 4.0 ppm with a standard deviation of 4.8 ppm. Conversely, external calibration was used with eq 2 and the aid of an internal lock mass (as discussed above for exact mass measurement of precursor ions). In this case, the remaining parent ion (or the ^{13}C peak if no ^{12}C peak remained) was used as the lock mass for the electric field term correction (Figure 2b). The observed errors in mass are given in Table 3 and were, as in the case of the precursor ions, consistently less than 2 ppm. The measurements had an absolute mean error of 0.7 ppm with a standard deviation of 1.0 ppm.

Effect of Apodization Functions

In addition to calibration, several other data processing parameters were examined in order to provide an optimal set of conditions for obtaining exact mass measurements. First, prior to fast Fourier transformation of the data, apodization was performed on the data. For all of the aforementioned mass measurements, a Hamming function was utilized; however, several other apodization functions were examined with eight of the compounds. Again, three measurements were conducted for each compound resulting in 24 total measurements. Besides the Hamming, Gaussian, Hanning, 3-term Blackman Harris, Triangle, and Kaiser Bessel functions were examined. The use of these six apodization functions resulted in mass measurement error(s) which were comparable. Furthermore, all of them produced approximately a 1 ppm absolute mean error and standard deviation (Table 4). The fact that there was little difference may have been due to the fact that the mass errors were quite low overall.

Peakfit Model

Another important aspect of obtaining reliable exact mass measurements is the selection of the center of the peak. Recent [24] FTICR quantitation studies have shown that the proper peakfitting algorithm can be quite important. There are several peak picking methods which are commonly used for this determination and four models were examined with the eight compounds mentioned above (three measurements/compound). The results of this study are presented in Table 5 and again, indicate that there was little difference in the observed mass errors produced between these methods. Furthermore, the absolute mean errors ranged from 1.0 to 1.2 ppm. There was, however, a clear improvement in both the mass accuracy and precision using any of the peak models versus none at all (i.e., using the maximum point on the peak to determine peak mass). Nevertheless, it is important to note that given the data set size and degree of zerofilling, approximately eight points defined each peak, and it is possible that less improvement with the peak picking algorithms versus simply using the maximum point might have resulted if the peaks were defined by more points.

Conclusions

The primary focus of this study was to examine the reliability of FTICR in obtaining exact mass measurements for a series of compounds using several different calibration methods and data processing algorithms.

Table 4. Exact mass measurement as a function of apodization—observed errors[a]

Apodization function	Absolute mean error	Standard deviation	rms error
3-Term Blackman Harris	0.5 mmu (1.1 ppm)	0.6 mmu (1.3 ppm)	0.6 mmu
Gaussian	0.5 mmu (1.0 ppm)	0.6 mmu (1.3 ppm)	0.6 mmu
Triangle	0.4 mmu (0.9 ppm)	0.5 mmu (1.2 ppm)	0.5 mmu
Hamming	0.4 mmu (1.0 ppm)	0.5 mmu (1.2 ppm)	0.5 mmu
Hanning	0.4 mmu (1.0 ppm)	0.5 mmu (1.3 ppm)	0.6 mmu
Kaiser Bessel	0.5 mmu (1.1 ppm)	0.6 mmu (1.5 ppm)	0.6 mmu

[a]24 measurements were made using eight compounds (three measurements/compound).

Table 5. Exact mass measurements as a function of peakfit model - observed errors[a]

Peakfit model	Absolute mean error	Standard deviation	rms error
No peakfit	0.9 mmu (1.6 ppm)	1.0 mmu (1.7 ppm)	1.0 mmu
Quadratic	0.5 mmu (1.1 ppm)	0.6 mmu (1.5 ppm)	0.6 mmu
3-pt quadratic	0.5 mmu (1.0 ppm)	0.6 mmu (1.4 ppm)	0.6 mmu
Lorentzian	0.6 mmu (1.2 ppm)	0.7 mmu (1.6 ppm)	0.7 mmu
3-pt Lorentzian	0.5 mmu (1.1 ppm)	0.6 mmu (1.4 ppm)	0.6 mmu

[a]24 measurements were made using eight compounds (three measurements/compound).

This approach differs somewhat from that employed by Sack et al., [6] where one compound was utilized in obtaining many measurements for several ions of different *m/z*. Furthermore, in that study, a magnetic sector instrument was utilized in developing a procedure for evaluating the accuracy and precision of exact mass measurements.

Both internal calibration methods examined in this study were found to produce mass measurements with high accuracy and precision as seen in the average absolute errors, the associated standard deviation, and the rms errors observed. Furthermore, the tandem use of ESI and MALDI for internal calibration (MITS) provides the potential for reliable exact mass measurements with higher throughput. During the course of this study, it became apparent that this technique was especially amenable to obtaining exact mass measurements for large numbers of samples, because no optimization of the analyte-calibrant solution was required. The ratio of ions could be controlled in the cell (rather than in solution) using suspended trapping [25] or by changing the ion transfer conditions. This was especially useful with samples containing small quantities of unknown. In contrast, conventional calibration via an internal standard was found to be more practical when performing mass measurements for only one or two samples, because of the extended initial setup required with MITS (setting up both ionization techniques).

External calibration was found to produce greater error(s) with exact mass measurements although there was a great deal of deviation in the data. At times, the observed error was less than 2 ppm, while at other times it was found to be greater than 10 ppm. This lack of consistency did not appear to be due to any drift in the magnetic field as indicated by the marked improvement in the errors when a single-point internal adjustment of the electric field term was employed. Furthermore, the use of external calibration with an internal lock mass produced error(s) (consistently <2 ppm) which were quite comparable with those observed for both internal calibration methods discussed above. This technique was also utilized to obtain exact mass measurements of fragment ions produced from MS/MS with similar results.

Several different processing algorithms were also examined in obtaining these mass measurements. First, six different apodization functions were found to produce results with very little deviation. The same was found to be the case with four different peakfit models which were studied. The fact that the apodization function and the peakfit model made little difference was probably due to the fact that internal calibration was used and the observed errors were quite low. Furthermore, the slight difference in error(s) might have been magnified had greater overall error(s) been exhibited.

It is important to note that the samples examined in this study had been submitted for routine exact mass analysis and were relatively pure ($>90\%$?). It is possible that the analysis of less pure samples would result in lower overall mass accuracy and/or precision. Certainly more pronounced space-charge effects would be expected with the greater overall ion population in the cell. Finally, all of the work discussed was obtained using a 7 tesla instrument exhibiting excellent magnetic field homogeneity and very little drift. It is possible that a lower field instrument (or one lacking the same degree of homogeneity and/or stability) might not produce the same results.

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